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(54) METHOD OF PREVENTING AGGREGATION OF PROTEINS/PEPTIDES UPON REHYDRATION OR THAWING

METHODE ZUM VERHINDERN DER AGGREGATION VON PROTEINEN/PEPTIDEN BEI REHYDRATATION ODER AUFTAUEN

PROCEDE PREVENANT L'AGGREGATION DE PROTEINES/PEPTIDES LORS DE REHYDRATATION ET DE DECONGELATION

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EP 0 762 897 B1

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Description

Field of the Invention

[0001] The present invention relates to methods of preventing the formation of aggregates of various substances upon dehydration and rehydration and upon freezing and thawing. Compositions obtained thereby are also encompassed by the invention.

Background of the Invention

[0002] Storage and processing of a wide range of substances in a dehydrated or frozen form is necessary to retain activity, prevent degradation products from forming and to facilitate handling and transport. Unfortunately, upon rehydration or thawing, many substances tend to aggregate, thereby decreasing their effective concentration and often rendering them useless or forming harmful byproducts.

[0003] Various methods have been tried to prevent or eliminate such aggregation. For instance, detergents and chaotropic agents are often used to prevent aggregation of proteins in solution. These agents are thought to prevent aggregation mediated by hydrophobic interactions and thus are limited to prevention of aggregation due to this cause. See, e.g., Tanford and Reynolds (1976) Biochim. Biophys. Acta. 457:133; and Tanford, "The Hydrophobic Effect", 2nd Ed., Wiley, N.Y. (1980). Such agents may also not be suitable for use where the substances are to be formulated into therapeutic compositions as they may cause adverse reactions.

[0004] Aluminium salts in solution are in the form of a highly hydrated colloidal gel and carry a surface charge at any pH outside their isoelectric point. Since each colloidal particle carries the same charge, they mutually repel each other and thus naturally form a stable colloidal gel. When the hydration shell is removed (e.g., by freezing or drying) the particles can contact each other and the surface energy causes aggregation.

[0005] Trehalose, α-D-glucopyranosyl-α-D-glucopyranoside, is a naturally occurring, non-reducing disaccharide which was initially found to be responsible for protection of intact plant cells from desiccation. Trehalose has been shown to be useful in preventing denaturation of proteins viruses and foodstuffs during desiccation. U.S. Patent Nos. 4,891,319; 5,149,653; 5,026,566; Blakeley *et al.* (1990) Lancet 336:854-855; Roser (1991) Trends in Food Sci. and Tech. pp.166-169; Colaco *et al.* (1992) Biotechnol. Internat., pp. 345-350; Roser (1991) BioPharm. 4:47-53; and Colaco *et al.* (1992) Bio/Tech. 10:1007-1011.

[0006] In the field of protein purification it would be particularly useful to eliminate or prevent the tendency of, eg. proteins, to aggregate upon rehydration and thawing. This is especially important in the area of biopharmaceuticals where the proteins are often used as an ongoing basis of treatment. In the case where protein aggregates form and are injected into a patient, antibodies may form to the protein which diminish the effectiveness of the treatment. Thus, it would be useful to prevent aggregation of a wide variety of substances particularly those useful in medicine.

Summary of the Invention

[0007] The invention encompasses a method of reducing aggregation during dehydration and rehydration of a protein or peptide comprising the steps of adding to a solution or suspension of the protein or peptide an amount of trehalose sufficient to prevent or reduce aggregation upon rehydration; and dehydrating the solution or suspension.

[0008] The invention further encompasses rehydrating the protein or peptide to obtain a solution or suspension of the protein or peptide in a substantially non-aggregate a form.

[0009] The invention further encompasses a method of reducing aggregation of a protein or peptide in solution or suspension during freezing (and optionally thawing) comprising the steps of adding to the solution or suspension of the protein or peptide an amount of trehalose sufficient to prevent aggregation during freezing; and freezing the solution or suspension.

[0010] The invention further comprises the step of thawing the frozen solution or suspension to obtain a solution or suspension of the protein or peptide in a substantially non-aggregated form.

Detailed Description of the Invention

[0011] The present invention encompasses a method of reducing aggregation during dehydration and rehydration of a protein or peptide by adding to an eg. aqueous solution or suspension of the protein or peptide an amount of trehalose sufficient to prevent aggregation upon rehydration; and dehydrating the solution or suspension.

[0012] The invention further encompasses a method of reducing aggregation of a protein or peptide in an eg. aqueous solution or suspension during freezing and thawing comprising the steps of adding to the solution or suspension of the protein or peptide an amount of trehalose sufficient to prevent aggregation during freezing and thawing; and freezing

the solution or suspension.

[0013] The invention also relates to eg. aqueous compositions containing trehalose in order to prevent or reduce aggregation, including frozen and dehydrated compositions.

[0014] The term "aggregation" as used herein refers to the interaction of two or more molecules of a protein or peptide such that they no longer behave as monomers but as dimers, trimers or other multimeric forms. Reducing aggregation decreases the concentration of multimeric forms compared to proteins or peptides dehydrated and rehydrated or frozen and thawed in the absence of trehalose. A protein or peptide substantially free of aggregates or substantially nonaggregated is one which, upon rehydration or thawing, contains a.decreased amount of multimeric forms of the protein or peptide compared to a control lacking trehalose. Typically, trehalose prevents, the formation of all multimeric forms of the protein or peptide. In the case of growth hormone, for instance, the addition of trehalose prior to dehydrating or freezing can result in the elimination of all multimeric forms with the exception of dimers. The dimers are, however, reduced in comparison to a control.

[0015] In a preferred embodiment, the proteins or peptides suitable for use in the invention have medical utility. The proteins and peptides can be those which form multimers upon dehydration/rehydration and/or freezing/thawing. The method of formation of multimers or aggregates is not critical to the invention.

[0016] The protein or peptide may be derived from natural sources made by recombinant or synthetic means and include analogues, agonists and homologs. As used herein "protein" refers also to peptides and polypeptides. Such proteins include, but are not limited to, growth hormones, growth factors, insulin, monoclonal antibodies, interferons and interleukins. Preferably, the growth hormone is human growth hormone.

[0017] The dehydration step can be performed by any method known in the art including, but not limited to, lyophilization, drying at ambient conditions or drying under reduced vapor pressure. When drying at reduced vapor pressure, the temperature at which the drying occurs is preferably below the temperature at which degradation of the protein or peptide occurs.

[0018] The freezing step can be performed by any method known in the art including, but not limited to immersing in liquid nitrogen, placing in a freezer which may be at -4°C to -80°C, dry ice and alcohol freezing bath. The samples should be maintained at a temperature suitable to maintain the frozen state. Thawing the frozen sample can be by any means known in the art, for instance at room temperature or at an elevated temperature. If thawing occurs at an elevated temperature, the temperature should be below that which causes, denaturation or other chemical changes in the protein or peptide. Optimal freezing and thawing temperatures can be determined empirically. Such a determination is within the skill of one in the art.

[0019] Once the proteins or peptides have been dehydrated or frozen, they can be stored indefinitely. The dehydrated proteins or peptides can store well at ambient temperatures, although they may be stored at any temperature below that which causes denaturation or other chemical changes. The invention further includes rehydration of the dehydrated samples to obtain solutions and suspensions substantially free of aggregates of the protein or peptide. Rehydration may add at least an amount of water sufficient to restore the buffer composition of the original solution or suspension but may add any amount of water or buffer.

[0020] The methods of the present invention require that the trehalose be present in a amount sufficient to prevent or reduce aggregation of the protein or peptide upon rehydration or thawing. Such a determination will be made empirically and is well within the skill of one in the art. Preferably, trehalose is added in an amount to obtain a final concentration of from about 1% to 50% (w/v). More preferably, trehalose is added in an amount to obtain a final concentration of from about 5% to 25% (w/v).

[0021] Trehalose is available from a variety of suppliers. Preferably the grade of trehalose used is ANALAR reagent, molecular biology or pharmaceutical grade. In the case of medicinal compositions the trehalose preferably meets the good manufacturing practice (GMP) standards set by the Food and Drug Administration (FDA).

[0022] Interestingly, the amount of trehalose found to be effective at preventing aggregation cannot be directly extrapolated from the amount of trehalose effective in preventing desiccation damage. For instance, work presented in United States Patent No. 4,891,319 showed that amounts of trehalose as low as 1% w/v in a protein solution could prevent desiccation damage to proteins such as Factor VIII. The Examples presented herein show that 15% w/v is necessary to prevent aggregation of a protein.

[0023] The following examples are meant to illustrate the invention.

Example 1

Effect of Trehalose on Aggregation of Biological Molecules

[0024] Protein formulations may undergo modification by a number of mechanisms including deamidation, oxidation and aggregation, the principle causes of human growth hormone (hGH) degradation. Deamidation and oxidation are considered collectively as chemical degradation. To date there is little evidence of any effect of these chemical degra-

dation products on biopotency. Pearlman and Bewly (1993) In: Wang and Pearlman eds. Stability and Characterization of Protein and Peptide Drugs, pp. 1-58, Plenum Press, New York.

[0025] Aggregation is the principle problem affecting hGH and other protein formulations used as biopharmaceuticals and may reduce biopotency. Soluble or insoluble aggregates can form as a result of both covalent and non-covalent interactions. A variety of stresses such as heating, freezing or agitation may induce aggregation. Whilst a visible insoluble aggregate may render a parenteral product unuseable, the major problem is the induction of an unwelcome immune response in the subject (Pearlman and Bewley, 1993). This is particularly detrimental where the protein formulations such as hGH are administered parenterally and in multiple doses.

[0026] The following experiment was performed to determine whether or not trehalose affected the aggregation of proteins. Samples of hGH (5 mg) were dried from 200 µl containing 15% trehalose, 5 mM $Na_2HPO_4 - 2H_2O$ adjusted to pH 7.4 with H_3PO_4 (formulation A). Two control samples were prepared: 5 mg hGH dried from 200 µl sodium phosphate buffer pH 7.4 (formulation B); and 5 mg hGH dried from 200 µl sodium phosphate buffer pH 7.4, 5 mg glycine, 25 mg mannitol (formulation C). These formulations were dried for 20 hours in a vacuum drier at a pressure of 30 millitorr and a shelf temperature of 40°C. They were subsequently sealed under vacuum in standard pharmaceutical serum vials with rubber closures and a crimped aluminium seal.

[0027] Following storage at 40°C in a dry incubator, samples were rehydrated with deionised water and analysed by reverse phase and size exclusion high performance liquid chromatography to determine chemical degradation and aggregation respectively according to the method described by Pikal et al. (1991) Pharm. Res. 8:427-436. These results are presented in Table 2.

[0028] Formulation A was subsequently re-analysed and compared with a conventionally freeze-dried essentially as described in Pikal et al. (1991) equivalent formulation (formulation D). These results are presented in Table 2.

Results

[0029] An accelerated aging protocol of four weeks at 40°C was utilized to assess stability and aggregation. The formulation containing trehalose performs very well under these conditions. No chemical degradation was observed and the limited aggregation detected was restricted to dimer formulation (Table 1, lines 1-4). The absence of high molecular weight aggregates is significant.

[0030] Two hGH controls were formulated, one without a stabilizing excipient (B) and one containing glycine and mannitol, that was similar to commercial formulations (C) (Table 1, lines 5-6). These formulations suffered from considerable chemical degradation and aggregate formation, both dimer and higher molecular weight. The values for the glycine. mannitol formulation were comparable with results from a previous study in which a similar formulation was freeze-dried (Table 2, line 7, Pikal, *et al.* 1991). When the stability of formulation A was compared with that of a freeze-dried equivalent (formulation D), no difference in terms of 40°C stability was observed (Table 2, lines 1-6). In Tables 1 and 2 chemical degradation is measured by the area under the curve represented by the deamidated protein.

[0031] Thus the hGH formulations containing trehalose, either dried at 40°C or freeze-dried, have been shown to be considerable improvements on previous formulations.

Table 1

Summary of hGH Stabilization and Aggregation Data (Part 1)					
Line	**Formulation**	**Treatment**	**% Chemical Degradation**	**% Aggregation Dimer**	**% Aggregation High Mol. Weight**
1	A	pre-dry	3.1	0.4	0.003
2	A	post-dry	3.3	0.6	0.06
3	A	2wk., 40°C	3.5	0.9	0.02
4	A	4wk., 40°C	3.4	1.1	0.002
5	B	4wk., 40°C	11.1	6.9	2.1
6	C	4wk., 40°C	8.2	2.2	0.8

Table 2

Summary of hGH Stabilization and Aggregation Data (Part 2)				
Line	**Formulation**	**Treatment**	**% Chemical Degradation**	**% Aggregation**
1	A	initial	4.15	0.66

Table 2 (continued)

Summary of hGH Stabilization and Aggregation Data (Part 2)				
Line	**Formulation**	**Treatment**	**% Chemical Degradation**	**% Aggregation**
2	A	2wk., 40°C	4.16	0.92
3	A	4wk., 40°C	4.25	1.04
4	D	initial	4.05	0.71
5	D	2wk., 40°C	4.09	0.86
6	D	4wk., 40°C	4.17	0.92
7	E	4wk., 40°C	8.2	3.0

Claims

1. A method of reducing or preventing aggregation during dehydration and rehydration of a protein or peptide, the method comprising:

   adding to a solution or suspension of the protein or peptide an amount of trehalose sufficient to prevent or reduce aggregation upon rehydration; and
   dehydrating the solution or suspension.

2. A method according to claim 1, further comprising rehydrating the protein or peptide to obtain a solution or suspension of the protein or peptide in a substantially nonaggregated form.

3. A method according to claim 1 or claim 2, wherein the dehydrating comprises lyophilization, drying at ambient conditions or drying under reduced vapor pressure.

4. A method of reducing or preventing aggregation of a protein or peptide in solution or suspension during freezing, the method comprising:

   adding to the solution or suspension of the protein or peptide an amount of trehalose sufficient to reduce or prevent aggregation during freezing; and
   freezing the solution or suspension.

5. A method according to claim 4, further comprising thawing the solution or suspension to obtain a solution or suspension of the protein or peptide in a substantially nonaggregated form.

6. A method according to any preceding claim, wherein the protein or peptide is a hormone, growth factor, insulin, monoclonal antibody, interleukin or interferon.

7. A method according to claim 6, wherein the protein or peptide is human growth hormone.

8. A method according to any preceding claim, wherein the amount of trehalose gives a concentration thereof of from 1% to 50% (w/v).

9. A method according to claim 8, wherein the concentration is from 5% to 25% (w/v).

Patentansprüche

1. Verfahren zur Verringerung oder Verhinderung von Aggregation bei der Dehydratation und Rehydratation eines Proteins oder Peptids, wobei das Verfahren umfaßt:

   Zugabe zu einer Lösung oder Suspension des Proteins oder Peptids einer Menge an Trehalose, die ausreicht, um die Aggregation nach Rehydratation zu verhindern oder zu verringem, und
   Dehydratation der Lösung oder Suspension.

2. Verfahren nach Anspruch 1, ferner umfassend die Rehydratation des Proteins oder Peptids, um eine Lösung oder Suspension des Proteins oder Peptids in einer im wesentlichen nicht-aggregierten Form zu erhalten.

3. Verfahren nach Anspruch 1 oder Anspruch 2, wobei die Dehydratation eine Lyophilisierung, Trocknung unter Umgebungsbedingungen oder Trocknung unter verringertem Dampfdruck umfaßt.

4. Verfahren zur Verringerung oder Verhinderung von Aggregation eines Proteins oder Peptids in Lösung oder Suspension beim Einfrieren, wobei das Verfahren umfaßt:

   Zugabe zu der Lösung oder Suspension des Proteins oder Peptids einer Menge an Trehalose, die ausreicht, um die Aggregation beim Einfrieren zu verringern oder zu verhindern, und
   Einfrieren der Lösung oder Suspension.

5. Verfahren nach Anspruch 4, ferner umfassend das Auftauen der Lösung oder Suspension, um eine Lösung oder Suspension des Proteins oder Peptids in einer im wesentlichen nicht-aggregierten Form zu erhalten.

6. Verfahren nach irgendeinem der vorhergehenden Ansprüche, wobei das Protein oder Peptid ein Hormon, Wachstumsfaktor, Insulin, monoklonaler Antikörper, Interleukin oder Interferon ist.

7. Verfahren nach Anspruch 6, wobei das Protein oder Peptid Humanwachstumshormon ist.

8. Verfahren nach irgendeinem der vorhergehenden Ansprüche, wobei die Menge an Trehalose eine diesbezügliche Konzentration von 1 % bis 50 % (Gew./Vol.) ergibt.

9. Verfahren nach Anspruch 8, wobei die Konzentration 5 % bis 25 % (Gew./Vol.) beträgt.

Revendications

1. Procédé pour réduire ou empêcher une agglomération pendant une déshydratation et une réhydratation d'une protéine ou d'un peptide, le procédé comprenant les étapes consistant à:

   ajouter à une solution ou une suspension de la protéine ou du peptide, une quantité de tréhalose suffisante pour empêcher ou réduire l'agglomération lors d'une réhydratation; et
   déshydrater la solution ou la suspension.

2. Procédé selon la revendication 1, comprenant de plus la réhydratation de la protéine ou du peptide pour obtenir une solution ou une suspension de la protéine ou du peptide sous une forme sensiblement non agglomérée.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel la déshydratation comprend une lyophilisation, un séchage dans des conditions ambiantes ou un séchage sous une tension de vapeur réduite.

4. Procédé pour réduire ou empêcher l'agglomération d'une protéine ou d'un peptide en solution ou en suspension pendant la congélation, le procédé comprenant les étapes consistant à:

   ajouter à la solution ou la suspension de la protéine ou du peptide, une quantité de tréhalose suffisante pour empêcher ou réduire une agglomération lors d'une réhydratation; et
   congeler la solution ou la suspension.

5. Procédé selon la revendication 4, comprenant de plus la décongélation de la suspension pour obtenir une solution ou une suspension de la protéine ou du peptide sous une forme sensiblement non agglomérée.

6. Procédé selon l'une quelconque des revendications précédentes, dans lequel la protéine ou le peptide est une hormone, un facteur de croissance, l'insuline, un anticorps monoclonal, l'interleukine ou l'interféron.

7. Procédé selon la revendication 6, dans lequel la protéine ou le peptide est une hormone de croissance humaine.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel la quantité de tréhalose donne une

concentration de celle-ci de 1% à 50% (poids/volume).

9. Procédé selon la revendication 8, dans lequel la concentration est de 5% à 25% (poids/volume).